Oral administration of Factor VIII in lipid vesicles

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The prevention or treatment of severe bleedings in patients with haemophilia A presently depends on intravenous administration of partially purified preparations of the missing coagulation Factor VIII. In spite of the revolutionary breakthrough that came in the treatment of haemophiliacs with the introduction of suitable Factor VIII preparations for clinical use, this therapy still presents a variety of problems, not the least of them being the recurrent injections themselves. Oral administration of these Factor VIII preparations is useless due to extensive degradation of the protein in the gastrointestinal tract. This breakdown may be overcome to a certain extent if the protein is packed in liposomes, which may decrease exposure to the digestive proteolytic enzymes. It has been reported that liposome entrapped proteins are capable of entering intact cells[1], and insulin loaded liposomes administered orally to diabetic rats can cause a fall in the blood glucose level[2].

FACTOR VIII LOADED LIPOSOMES

Liposomes are artificial structures of multilamellar concentric bilayers of phospholipids that form spontaneously upon suspending lipids in water[3]. When liposomes are prepared in the presence of aqueous solution of a protein, 5-15% of the protein may become entrapped in the interstices between the bilayers[4]. Because Factor VIII has been reported to interact hydrophobically with phospholipids[5], we thought that it might be possible to preferentially absorb Factor VIII on phospholipids and therefore obtain liposomes with a much higher Factor VIII content than is usually obtained with non-lipid binding proteins. In spite of the fact that liposomes and their entrapped proteins are also susceptible to gastrointestinal breakdown, we thought that the specific binding of Factor VIII to the lipids and the much higher protein loading of the liposome, would produce an increased resistance to proteolytic attack and improve the chances for the protein to

reach the target undamaged.

The preparations to be used for oral administration were made from egg yolk lecithin containing 5-10% of phosphatidic acid and solutions of either Factor VIII concentrate or cryoprecipitate. Usually 50 mg of phospholipid were used per ml of Factor VIII solution. A detailed description of the procedure has been published[6].

The fraction of non-entrapped protein can be measured in the supernatant after centrifuging the liposomes at 50,000 x g for 10 minutes. This fluid contained 19-32% of the original Factor VIII added and usually more than 80% of the fibrinogen present in Factor VIII preparations. This strongly suggests that the liposome preparations are indeed preferentially enriched with Factor VIII, presumably due to the strong interaction between Factor VIII and the lipids. Direct measurement of the entrapped protein has so far been hampered by the presence of phospholipids. It is not to be expected, however, that significant denaturation of Factor VIII occurs because carrying out the same manipulations with a Factor VIII solution in the absence of lipids causes less than 5% inactivation.

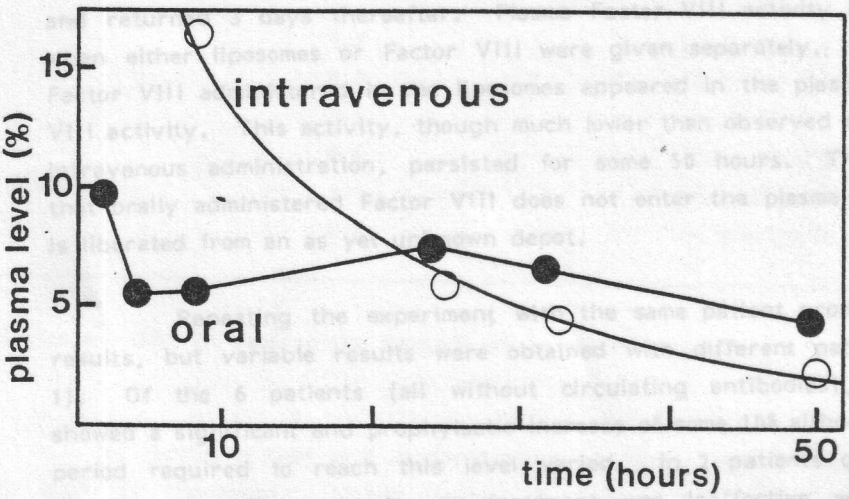

Figure 1 Factor VIII activity in plasma after oral and intravenous administration of Factor VIII to a patient with severe haemophilia. Concentrations were determined according to Veltkamp[7] and are expressed as a percentage of Factor VIII concentration in pooled normal plasma (n = 30)

Each sample was tested 4 times. SEM did not exceed 9% of value observed. At zero time 800 units of Factor VIII were given orally or intravenously. Factor VIII concentration in the untreated subject is less than 0.5% of normal. Concentration after intravenous administration measured before 9 hours were between 30% and 15% and have been omitted from the graph.

ORAL ADMINISTRATION

In a first experiment Factor VIII loaded liposomes were given before breakfast to a patient with severe haemophilia A (mean Factor VIII level when not on treatment less than 0.5% of normal; no circulating antibodies). The results are shown in Figure 1. Plasma levels after intravenous administration of the same amount of Factor VIII (not entrapped in liposomes) are also shown. The patient had haematuria before ingestion of the liposome preparation; this disappeared on the day of the experiment and returned 3 days thereafter. Plasma Factor VIII activity did not rise when either liposomes or Factor VIII were given separately. Some of the Factor VIII administered in the liposomes appeared in the plasma as Factor VIII activity. This activity, though much lower than observed shortly after intravenous administration, persisted for some 50 hours. This suggests that orally administered Factor VIII does not enter the plasma directly but is liberated from an as yet unknown depot.

Repeating the experiment with the same patient produced similar results, but variable results were obtained with different patients (Table 1). Of the 6 patients (all without circulating antibodies), 2 patients showed a significant and prophylactic increase of some 10% although the time period required to reach this level varied. In 2 patients only a minor increase was observed whereas treatment was ineffective with 2 other patients. It is to be noted that in no instance could any significant increase in serum phospholipid concentration be detected. This suggests that the Factor VIII containing liposomes do not enter the blood as such, which may limit the chances for new antibodies to form.

DISCUSSION

Although it is hardly possible to draw conclusions from

comparable to, or even longer than, that observed after intravenous administration of the same amount of Factor VIII. We have no explanation for the fact that some patients show little or no response. The efficacy of the treatment will certainly depend upon protection from, and time of exposure to, proteolytic digestion in the gastrointestinal tract. In this respect it is of interest that upon oral administration of insulin loaded liposomes to fasted and non-fasted rats, we only observed a large drop in blood glucose level in the group of fasted rats (Figure 2). A difference in resorption time may be responsible for this effect. Whether or not the resistance to digestive enzymes can also be improved by altering the phospholipid composition still needs to be investigated.

Table 1 Oral administration of 1300 U Factor VIII concentrate, trapped in 7 g liposomes (PC : PA = 9 : 1)

Patients	F VIII % t = o	Maximal F VIII % t = x
1	0.7	10.0 (x = 1.5 h)
2	3.8	10.2 (x = 4 h)
3	0.5	1.4 (x = 2 h)
4	0.6	1.2 (x = 2 h)
5	5.0	no change in 5 h
6	0.6	no change in 5 h

Patients 4-6 did not receive the material on an empty stomach.

We conclude that oral administration of Factor VIII in liposomes can lead to therapeutic plasma levels of Factor VIII, although the circumstances required for a successful treatment are not yet completely understood. A more extensive investigation is required in which particular attention should be paid to possible undesired antigenic reactions, although at present we have no indications that these occur.

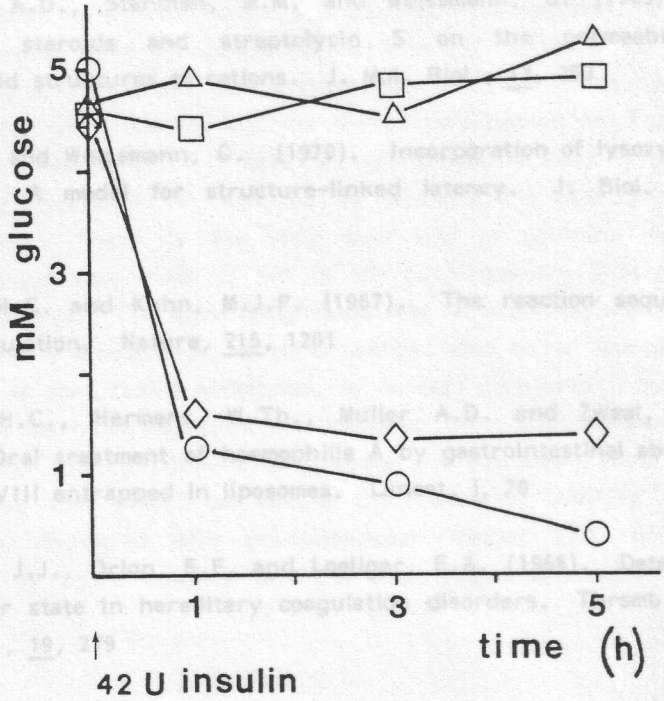

Figure 2 Plasma levels of glucose after oral administration of liposome entrapped insulin to fasted and non-fasted rats. —□-□—, non-fasted rats; -◇-◇—, fasted rats; —△-△—, non-entrapped insulin; —○-○-, intraperitoneal administration of non-entrapped insulin

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Discussion

Dr. Austen: Could Professor Zwaal tell us how he knows he has got so much Factor VIII entrapped in the liposome. I believe he stated 60-80 per cent. We have been preparing these liposomes over a long period now and my figure is nearer 1 per cent, which is rather different. Incidentally, we have treated one Factor IX deficient patient and two rabbits so far, with a total failure on all counts.

Prof. Zwaal: Had we studied patient No. 6 first, we would probably have dropped the whole thing. It was just a matter of luck that we had the right patient or the good patients first.

I do not know what Dr. Austen's phospholipid composition is. It is absolutely necessary to have a negative surface charge. Normally we use Ac-lecithin containing about 10 per cent phosphoteric acid, or in between 5 and 10 per cent, and the negative surface charge is required for Factor VIII to bind to the phospholipd. It does not spontaneously bind to pure lecithin. The other question - how do we measure the entrapment? There are two ways. There is the very easy way of spinning down liposomes and measuring how much is left in the supernatant. That is a very easy way of doing it. The more complicated way is when one wants to know how much is really entrapped. In other words, what is not measured in the supernatant, is that really entrapped, or is that denatured. Initial experiments in which we have degraded the liposomes, with phospholipases, show indeed that the total procedure to prepare the liposomes does not result in a loss that is more than 5 per cent of the Factor VIII activity. So where liposomes are degraded with phospholipases, Factor VIII can be measured because it is again released and the amount that was entrapped can be measured.

Dr. Austen: So that in fact one can take the liposomes, get zero Factor VIII in an assay, then lyse these liposomes, and in the following assay get 60 per cent of the starting material.

Prof. Zwaal: That is correct.

Prof. Stewart: Does it make any difference what sort of Factor VIII preparation is used?

Prof. Zwaal: We have tried two preparations, the cryoprecipitate and the Factor VIII concentrate.

Prof. Stewart: What concentrate?

Prof. Zwaal: I think it was Swiss in origin but I am not quite sure.

Prof. Stewart: Does it make any difference?

Prof. Zwaal: No. Not in these experiments - at least in the first patients, who always respond successfully.

Dr. Ludlam: Has Prof. Zwaal tried the control experiment giving the liposomes without Factor VIII and then measuring the Factor VIII level in the blood?

Prof. Zwaal: Of course we have. The result is nil.

Dr. Tuddenham: What sort of recovery does Prof. Zwaal get? How much Factor VIII does he put into the vessel in which he prepared the liposomes and how much gets into the patient?

Prof. Zwaal: Assuming a trap of between 60 and 80 per cent when we introduce 1300 units of Factor VIII. These are really entrapped units, so we started with approximately 1700 or 1800 units of Factor VIII of which about 1300 units were entrapped. But the first experiment was done with 800 units.

Dr. Tuddenham: How much got into the patient?

Prof. Zwaal: We have not really measured that. The therapeutic level above 5 per cent is maintained for at least the same time or longer than the level reached with an intravenous injection.

Dr. Pepper: As Prof. Zwaal states, specific entrapment is usually rather inefficient, and 60 per cent does imply some kind of specific affinity. Is there any other evidence, apart from those numbers, of the nature of the interaction which you postulated between VIIIC and the phospholipids in this situation?

Prof. Zwaal: Normally given pure labelled Factor VIII, one can do binding constants between Factor VIII and phospholipids. As I stated, this depends on the negative surface charge which is very similar to Factor V.

Dr. Pepper: Is it reported by anybody else in this sort of situation?

Prof. Zwaal: That there is a binding of Factor VIII in the lipids? I think there are a number of indications from the literature, but as far as I can recall there is no separate article devoted to that.

Prof. Bloom: Dr. Pepper is asking whether Prof. Hemker has repeated his earlier experiments with the use of phospholipids that they are actually using in the liposomes.

Prof. Zwaal: No, he has not. But I showed very early experiments. But, when we take the pure Factor VIII preparations as are presently being prepared, we can label them and measure binding between these Factor VIII and the lipids, and we find only high affinity when the lipid is negatively charged.

Dr. Birch: How stable are liposomal preparations because in practical terms if they are not likely to prove stable on the bench or in the refrigerator for some time, they may not be of much use.

Prof. Zwaal: Normally a liposome preparation is stable when it is not sterilised for about 2 to 3 days in a refrigerator. It might be interesting to look at whether one can lyophyllise the complete liposomal preparation, or just freeze it and see whether it can be used again without any loss of activity in the Factor VIII. But this has not been done. Normally the preparation is stable for about 2 to 3 days.

Dr. Delamore: Has anyone tried giving it with the appropriate lipolytic enzymes to see whether that will promote absorption?

Prof. Zwaal: Why should one promote absorption?

Dr. Delamore: Prof. Zwaal did suggest that if it was not given on a fasting stomach he did not get the appropriate action from lipolytic enzymes.

Prof. Zwaal: The idea behind it is very simple, that there is an enhanced degradation due to the production of lipolytic enzymes when the stomach is not empty. But one can simply degrade the liposomes by lipolytic enzymes. Of course one can. And then the Factor VIII is liberated.

Prof. Bloom: Then presumably it would not get absorbed. Can Prof. Zwaal postulate the mechanisms of absorption?

Prof. Zwaal: I have no idea. I can speculate on it. It has been shown that normally most of the lecithin which is administered orally is degraded, and the fatty acids of the lecithin appear for instance in triglycerides in the blood. It has also been shown that under certain conditions lecithin can be taken up completely as a complete molecule. But in this case we have measured the possibility of a rise in the serum levels of lecithin in the plasma and we could not detect any, whereas we could have when most of the lecithin would have been liberated to the plasma.

Prof. Bloom: Presumably this goes into the portal system, into the chyle. Does it get a deposit there in the lymph nodes?

Prof. Zwaal: The fact that it is maintained, at least in the successful experiments, for about 3 days at a level of between 5 and 10 per cent simply suggests that it is liberated from a depot as yet unknown.

Prof. Bloom: Which is why I asked whether the depot could possibly be in the lymphatic system.

Prof. Zwaal: It could be. It could be anywhere.

Dr. Preston: Has there been an opportunity to test the liposomes in patients with vW disease?

Prof. Zwaal: That is a very good question. We are really willing to do this, but as I mentioned this is only a very small sideline in our laboratory. We do not normally do research into haemophilia.

Prof. Bloom: This is a very exciting possibility. I really hope that it does develop.